NEWS & VIEWS FEATURE

MICROBIAL SCIENCES

The superficial life of microbes

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The social activities and organization of bacteria are crucial to their ecological success. But it is only in recent years that we have begun to study these secret societies.

Most surfaces on this planet teem with microbial life, creating ecosystems of diverse organisms that flourish in slimy beds of their own making. The plaque encrusting our teeth, the slippery coating on river stones, the gunge clogging up water pipes or infected wounds: these are just a few examples of the microbial 'biofilms' that form anywhere there is a surface with a little moisture and some nutrients. Although microbes by and large live in such biofilm communities, most of our understanding of their physiology stems from experiments using liquid cultures of dispersed, free-swimming 'planktonic' cells. In the past decade, however, the number of studies performed on surface-associated microbes has increased dramatically. Today, we recognize that most, if not all, microbial species can form biofilms. The physiological differences between free-living individuals and communal biofilm-associated cells are becoming apparent, as are the regulatory mechanisms that underlie the switch between these two lifestyles.

The diverse universe of biofilms

When individual bacterial cells encounter a surface under conditions propitious for growth, they almost invariably undergo dramatic lifestyle changes. The nomadic single cells settle down on the surface, where they divide and establish a sedentary yet remarkably diverse community (Fig. 1a). These are communities in the sense that we humans organize ourselves into communities with division of labour — as the surface-associated population grows, the biofilm becomes increasingly sophisticated in its activities, with individual cells taking on specific tasks. As a result, biofilms can develop intricate architectures; striking mushroom-like structures can bloom on submerged surfaces, and aerial projections sprout from surfaces exposed to the air (Fig. 1b,c). The diversity of biofilm architectures is akin to a miniature coral reef, with their structures differing enormously depending on the species present and the environmental conditions.

Natural biofilms nearly always harbour a multitude of microbial species — the region around a single tooth, for example, is often sheathed in a community of several hundred species[1]. Within such microbial ecosystems, species richness may ensure stability of the ecosystem in the face of changing environmental conditions, very much as species richness is thought to aid the stability of macroscale ecosystems such as a tropical rainforest.

Even in the context of artificial, single-species biofilms, strain diversification seems to be the rule. Unlike the growth of bacteria in liquid cultures, which produce homogeneous populations of genetically identical cells, growth in biofilms generates a large amount of genetic diversity[2]. How can a single cell, with a single genetic complement, give rise to a biofilm population in which the individual cells are genetically different from one another? The simplest explanation may be that in any biofilm, individual cells are stuck in the same place, attached to their neighbours and the slime that surrounds them, so their access to nutrients will vary as gradients form within the biofilms through metabolic activity. As a consequence, many microniches are likely to arise, as are random spontaneous mutants that can exploit those microniches. As various mutants grow in their location, the result will be a biofilm containing a multitude of genotypes. This genetic diversity within a strain may provide a sort of insurance, as the population can adapt better to sudden environmental changes than can a genetically homogeneous population.

To understand these minute ecosystems, it is necessary to be able to grow, observe and manipulate biofilms in the laboratory. This is accomplished using transparent flow cells, where bacteria attach to a glass surface and are continuously fed fresh nutrients[3]. The ensuing biofilm growth can be followed using confocal laser microscopy. In such flow cells,
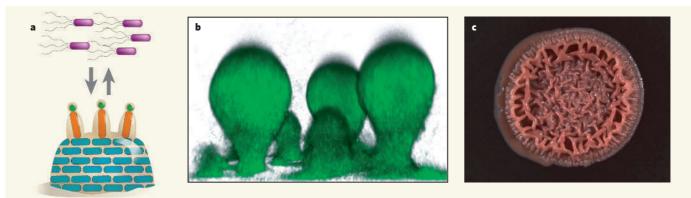

Figure 1 | Biofilm formation and architecture. a, Individual bacteria undergo a reversible lifestyle switch between a nomadic and a sedentary existence. In the process, they lose motility and become enclosed in a gooey extracellular matrix. As the community grows, different cell types appear as the original strain diversifies to allow cells to take on different tasks or to exist in different 'microniches', shown by differently coloured cells. **b,** These mushroom-like structures are characteristic of many submerged biofilms. In this *Pseudomonas aeruginosa* biofilm, which was grown in a flow cell, they are about 150 μm high. **c,** A biofilm of *P. aeruginosa* grown on a semi-solid agar surface (about 10 mm in diameter). The colony displays aerial projections or wrinkling characteristic of an air-exposed biofilm.

experimental reproducibility is best obtained using single-species systems. As a consequence, many microbial species have been analysed as monocultures. Yet, in natural settings, these microbes nearly always grow in the context of multi-species communities. To approximate the natural setting more closely, investigators have begun to foray into systems containing two or more microbial species. Some potentially interesting multi-species model systems are poised for molecular analyses. Among these are mixed-species biofilms of the opportunistic pathogens *Pseudomonas aeruginosa* (a bacterium) and *Candida albicans* (a fungus)[4]; two bacterial species that colonize teeth, *Streptococcus gordonii* and *Veillonella atypica*[5]; and two species that occur in the soil environment around plant roots, *P. aeruginosa* and *Agrobacterium tumefaciens*[6].

Sticking together

Given the diversity of species that form biofilms, it is not surprising that the molecular mechanisms involved in biofilm development reflect the remarkable variety of the microbial world. Different species build biofilms differently, and many strains have multiple biofilm-formation pathways. For instance, some mutants of the soil bacterium *Pseudomonas fluorescens* that cannot make a biofilm when grown with glucose as the sole carbon source do form biofilms when the sole carbon source is glutamate[7].

So, do any general principles emerge? One feature that does seem to be common to all biofilms is that the cells secrete a 'matrix' to hold themselves in place and to provide a buffer against the environment. The make-up of each matrix is different, and, depending on the contributing species and environmental conditions, there can be various mixes of polysaccharides, proteins and even nucleic acids[8].

The rich diversity among the matrix components can be glimpsed by mentioning what is known about the polysaccharides secreted by just one species, *P. aeruginosa,* one of the most-studied organisms in terms of biofilm formation. Many strains of this bacterium have the capacity to extrude at least three types of extracellular polysaccharide, called alginate, Pel and Psl. Alginate was initially considered the major biofilm polysaccharide, but it now seems that it is a major matrix component only of the biofilms formed by unusual variants[9]. Pel and Psl are apparently more ubiquitous: the genes involved in the synthesis of Pel occur in all *P. aeruginosa* strains studied, whereas the genes associated with Psl are present in only some[10–12]. But even the Pel genes have diverse regulatory regions, suggesting that the various strains express this matrix component differently.

Lifestyle choices

Motile bacteria generally have flagella — corkscrew-like appendages that rotate to propel them. These structures occur only in the microbes' planktonic form, and it now seems
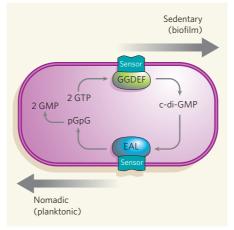

Figure 2 | The lifestyle switch. The second-messenger molecule c-di-GMP mediates the switch between nomadic and sedentary lifestyles. Many species of bacteria have a multitude of enzymes capable of making c-di-GMP from GTP (GGDEF proteins), and of breaking it down by hydrolysing it into GMP (EAL proteins). Overall, it seems that increased intracellular levels of c-di-GMP favour a sedentary existence, whereas reduced levels of the second messenger favour a nomadic existence.

that production of flagella and of extracellular matrix are mutually exclusive processes: bacteria give up the ability to move in order to settle down. The regulatory circuitry involved in this lifestyle switch is beginning to be mapped out. In *Bacillus subtilis*, for instance, the switch between flagellar synthesis and matrix production is controlled by the master gene regulator SinR (refs 13–15). This protein directly represses genes encoding matrix components and activates those encoding flagellar components (seemingly indirectly). SinR activity itself is antagonized by another protein, SinI, whose abundance and activity are modulated by changing environmental conditions. The inverse regulation of genes involved in motility and matrix components is apparent in several other organisms, including *E. coli, P. aeruginosa, Salmonella enterica* and *Vibrio cholerae*, although the specific regulatory molecules used have not been identified[16,17].

But how is the lifestyle switch thrown? An individual cell must have some way of recognizing that it is near a suitable surface, and of passing that information on to the master gene regulators and other effectors so that it can cease roaming and settle down. In several species, the messenger seems to be a small cytoplasmic molecule called bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP)[18] (Fig. 2). Low intracellular c-di-GMP concentrations are found in planktonic cells, but these levels rise once the cells have given up their nomadic lifestyle. Furthermore, it looks as though c-di-GMP can control the synthesis of diverse cellular components involved in both motility and matrix production in response to changing environments.

The synthesis and degradation of c-di-GMP

are carried out respectively by proteins that have structural domains possessing diguanylate cyclase and phosphodiesterase enzymatic activities (referred to as GGDEF and EAL domains, respectively, because of the highly conserved amino-acid sequences they contain). Genomic analyses of dozens of microbial genomes show that most bacteria harbour a multitude of these proteins — *Vibrio vulnificus*, an extreme example, has 66 GGDEF proteins and 33 EAL proteins[18]. Often, the GGDEF or EAL segment is fused to one of several types of environment-sensing domains, implying that the production and breakdown of c-di-GMP are controlled by the bacterium's surroundings.

The GGDEF and EAL proteins, and by extension c-di-GMP, are involved in many diverse cellular activities, but the mechanism by which c-di-GMP acts remains largely unknown. It may act to integrate the many external inputs sensed by GGDEF and EAL proteins, allowing this single messenger to instigate multiple indirect effects. Or perhaps there are small cellular 'compartments' where local fluctuations in the concentration of c-di-GMP cause independent outcomes. Initially, c-di-GMP was shown to function by activating the enzyme that synthesizes extracellular cellulose in *Gluconoacetobacter xylinus*. This activity occurs at what is known as the post-translational level, once the target protein, in this case the enzyme, has been synthesized[19]. By extension, investigators believed c-di-GMP acted exclusively at the post-translational level on enzymes in other bacteria. But it now seems that c-di-GMP can affect many processes, not only by acting post-translationally, but also by regulating gene expression. For example, it functions at the level of gene-transcription control in *P. aeruginosa*[20]. No gene-regulatory proteins that bind to c-di-GMP have yet been identified conclusively, so how this messenger passes on the external signal to the master regulators is unclear.
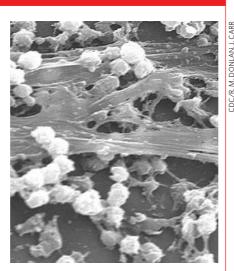
Even though the structure of c-di-GMP and its involvement in regulating cellulose synthesis in *G. xylinus* were reported[21] in 1987, it was only in the past few years, when it became apparent that c-di-GMP occurs in bacterial pathogens, that interest in this molecule really intensified. In this sense, the history of c-di-GMP research resembles the history of quorum sensing — the means by which bacteria detect the presence of others of their kind. As early as 1968, there were reports of what we now know as quorum sensing in the marine bacterium *Vibrio fischeri*[22]. But it was only in the mid-1990s that quorum sensing was recognized as a feature of many pathogens[23], leading to a flood of interest in this process. One has to wonder how many interesting molecular mechanisms have already been found in microbes but have yet to receive much attention because they are not being studied in pathogens.

Box 1   Biofilms in human health and disease

Environmental microbiologists and engineers have long recognized that biofilms form on surfaces — clogging pipework, for instance, or aiding water clean-up — but this fact has only recently been appreciated fully by those in the medical sciences. The intimate relationship between the human body and its resident microbes is now beginning to be elucidated.

Such biofilms are mostly beneficial. By colonizing our skin, teeth and the mucosal surfaces lining our gut and airways, thousands of different microbial species play an essential role in our nutrition and serve as a primary line of defence against invading pathogens. But although they are vital to our well-being, we know very little about the microbiota that thrive on and in our bodies. We scarcely know how to cultivate even a small percentage of these microbial inhabitants so as to be able to study them closely.

Some biofilms, however, are extremely harmful to us. Notably, foreign objects such as catheters and prostheses provide enticing surfaces for colonization, and microbes often quickly take up residence (for example, the image shows a *Staphylococcus aureus* biofilm found on an in-dwelling catheter, magnified 1,180 times). The resulting biofilms can become reservoirs for

systemic infection. Unfortunately, for reasons that remain poorly understood, biofilm-associated microbes are particularly impervious to many antimicrobial agents, so biofilm-related infections are difficult to treat. A major goal in this field is to develop therapeutic strategies that can control the undesirable growth of biofilms while leaving beneficial biofilms intact.

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decade have the tools of molecular biology been brought to bear on biofilms — a form that has immense biological significance. With recent technological developments that allow analysis of multi-species biofilms, the next decade should see further integration of molecular approaches into the study of biofilm biology, and significant progress in our understanding of more complex problems of bacterial social behaviour. At an applied level, we can hope to develop agents that control the biology of biofilms — to allow them to be analysed further, to treat biofilm infections, and to control biofilms for beneficial uses (Box 1). Clearly, much remains to be discovered about the diverse world of bacterial biofilms, and it seems the time is ripe for such discoveries.   ■

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Community relations

Once an advance party of bacteria has set up camp on a pristine surface, the cells can let each other know they are there by exuding quorum-sensing signals[23]. Planktonic bacteria use these chemical signals to regulate a variety of processes, depending on population density. But in many bacterial species the same signals are now known to be cues for biofilm development. This was first shown in *P. aeruginosa*, in which quorum-sensing signals control the expression of hundreds of genes throughout the genome[24,25]. In certain surroundings, mutants defective in quorum signalling are also defective in biofilm formation; for instance, they do not grow mushroom-like structures under conditions where these structures would normally form. The mutants still make biofilms, however, and in certain conditions where mushroom structures would not usually form, these biofilms are indistinguishable from those formed by unmutated strains. This observation may be due to the fact that the effects of the quorum-sensing mutations on biofilm formation are not apparent visually, or that quorum sensing is involved in specific steps in biofilm formation such as mushroom building. So it seems that biofilm formation is a social activity that is also governed by both genetic and environmental factors (reviewed in ref. 26).

An area that will see increasing attention involves the question of whether signalling in biofilm biology occurs between species. Multi-species biofilm model systems are now available to address such questions, and there is mounting evidence that bacteria do have systems that monitor and respond to quorum-sensing signals from other species. In some cases these systems are very specific, and in others there may be methods for sensing signals or cues produced by almost any member of the local microflora[27].

If different species can sense one another, how do they interact? Can biofilm communities encourage or discourage individuals in the planktonic community over entry to the biofilm? In *P. aeruginosa* biofilms, the quorum-sensing response results in the activation of a battery of potential defence systems, including a system for producing cyanide and one that releases antibiotics to attack other bacteria[25,28]. Whether this response does indeed constitute a defence against interlopers is being studied experimentally by tagging the biofilm bacteria and the planktonic bacteria with differently coloured fluorescent proteins (G. J. Balzer, M. H. Hentzer and M. R. Parsek, personal communication).

Outlook for the future

Surfaces afford a greater capacity for organization than do liquids, and so microbes encountering surfaces can form aggregates that begin to display some attributes of multicellularity. These groups of cells are held in place by an extracellular matrix and can use intercellular signalling for communication. In doing so, they develop intriguing features that we are only just beginning to understand.

Traditionally, microbes have been studied in their planktonic forms. Only in the past